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# FURTHER OBSERVATIONS ON A PLAGUE-LIKE DISEASE OF RODENTS WITH A PRELIMINARY NOTE ON THE CAUSATIVE AGENT, BACTERIUM TULARENSE.\*

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In a previous paper<sup>1</sup> one of us described a disease found in nature in California ground squirrels (*Citellus beecheyi* Richardson) which is experimentally transmissible to other rodents, and which in practically all susceptible animals gives rise to plague-like lesions. The following is the summary of the observations reported in the paper mentioned:

A disease which presents lesions very similar to those of plague has been found among ground squirrels.

The disease is readily transmitted to guinea-pigs, mice, rabbits, monkeys, and gophers, and plague-like lesions are produced in at least some of these animals. Rats are but moderately susceptible to the infection. Cats, dogs, and pigeons appear to be immune.

The disease may be transmitted artificially by subcutaneous, cutaneous (vaccination), nasal, and intraperitoneal inoculation. The mode of transmission in nature is unknown, but there is some experimental evidence to suggest that fleas may serve as carriers. The disease is probably not spread by mere contact of healthy with infected animals.

The infectious agent has not been isolated. On account of the number of species susceptible, it seems likely that it is bacterial, not protozoan.

The causative agent is present in the circulating blood, as well as in the various tissues where it causes focal lesions.

The thermal death point of the organism as it is found in the animal's body is between 55° C. and 60° C.

Several observations appear to indicate that the disease is a febrile one.

We wish to report certain observations based upon a larger experience with naturally infected ground squirrels, and upon considerable experimental work, including the cultivation of the organism that causes the disease.

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<sup>1</sup> *Pub. Health Bull. No. 43*, U.S. Public Health and Marine Hospital Service, Washington, 1911.

## THE DISEASE IN NATURALLY INFECTED SQUIRRELS.

*Size and sex.*—Of 42 squirrels found naturally infected with this disease 11 are noted as half grown, two as three-fourths grown, 25 as full grown, four size not stated. As to sex, 24 of these were males and 14 females; sex of four not stated.

*Lesions.*—A bubo is the most common lesion, though it is not constant. The affected gland is caseous and is frequently surrounded by a hemorrhagic area.

*Spleen.*—This organ is very much enlarged and is usually dark in color. A slaty appearance is not unusual. The essential lesions are whitish or yellowish caseous granules which vary in size from a pin point to perhaps 1 mm. in diameter.

*Liver.*—The only change noted in the liver is the presence of granules similar to those found in the spleen.

*Lungs.*—Lung lesions are rare. Granules have been noted in one case. In ground squirrels this rarity of lung lesions constitutes the most important anatomical difference between this disease and plague, since in the latter lung lesions are quite common.

## FREQUENCY OF VARIOUS LESIONS.

BUBO:	
Present.....	40
Absent.....	6
Single: inguinal.....	8
axillary.....	5
cervical.....	13
pelvic.....	6
Multiple: inguinal and pelvic.....	4
cervical and axillary.....	2
cervical and inguinal.....	2
SPLEEN LESIONS:	
Present.....	35
Absent.....	11
LIVER LESIONS:	
Present.....	21
Absent.....	25
LUNG LESIONS:	
Present.....	1
Absent.....	45

*Frequency.*—The frequency with which the disease is encountered varies with the localities from which squirrels are received. Certain

districts show a high percentage of this infection, while it is absent in others. In a series of 10,000 squirrels coming from various points between the Sacramento River on the north and the Tehachapi Mountains on the south, this disease was observed seven times, while plague infection was found 10 times.

#### PATHOGENICITY.

The results of additional experiments to determine the susceptibility of certain animals previously used and of some that had not been made the subject of investigation are presented here.

*Susceptibility of rats (*Mus norvegicus*).*—These rodents gave somewhat contradictory results in previous experiments; therefore we have made several observations on this subject. Large doses of infectious material given subcutaneously often result fatally. Small doses are less effective and inoculation by scarification usually fails to convey the disease to rats.

The following is a fair example of several experiments that we have performed: Six white rats were inoculated subcutaneously with a heavy emulsion made from the tissue of a guinea-pig dead of the disease. One died on the third day and two on the fifth day. They presented the lesions usually found in these rodents dead of this infection. The other three survived. Three white rats that were inoculated by the cutaneous method with the same material showed no evidence of illness.

Several experiments have been performed for the purpose of ascertaining whether there is any difference in the susceptibility of wild rats and of white ones. The most extensive one is presented here.

Series of rats, white and wild, were inoculated with various doses of emulsion made from the liver and spleen of a guinea-pig dead on the third day. Half a gram of each organ was ground in a mortar with sand and 10 c.c. of physiological salt solution. The emulsion was strained through cheesecloth and 1 c.c. given subcutaneously to each of two white and two wild rats. Each succeeding pair of each variety was given a dose one-tenth as large as that given to the preceding pair in the series. The results are set forth in the following table:

Two guinea-pigs were used for controls. One received 0.000001 c.c. and the other 0.000001 c.c. of the emulsion. Both died of the plague-like disease on the eighth day.

From this experiment it seems that the two varieties of rats are about equally susceptible and that extremely small doses of the infectious material are occasionally successful in conveying the disease to these rodents.

WHITE RATS			WILD RATS		
Weight of Rats, Grams	Dose of Emulsion	Day of Death*	Weight of Rats, Grams	Dose of Emulsion	Day of Death*
160	1 c.c.	9	250	1 c.c.	neg.
160	1 c.c.	neg.	280	1 c.c.	neg.
130	0.1 c.c.	7	260	0.1 c.c.	6
135	0.1 c.c.	7	230	0.1 c.c.	9
100	0.01 c.c.	neg.	195	0.01 c.c.	neg.
115	0.01 c.c.	neg.	160	0.01 c.c.	neg.
105	0.001 c.c.	neg.	170	0.001 c.c.	neg.
130	0.001 c.c.	neg.	160	0.001 c.c.	neg.
135	0.0001 c.c.	neg.	215	0.0001 c.c.	neg.
115	0.0001 c.c.	neg.	150	0.0001 c.c.	neg.
110	0.00001 c.c.	neg.	145	0.00001 c.c.	neg.
115	0.00001 c.c.	neg.	140	0.00001 c.c.	neg.
100	0.000001 c.c.	neg.	120	0.000001 c.c.	9
70	0.000001 c.c.	neg.	60	0.000001 c.c.	neg.
90	0.0000001 c.c.	11	175	0.0000001 c.c.	neg.
95	0.0000001 c.c.	neg.	275	0.0000001 c.c.	neg.

\* In each case where a rat died the diagnosis was verified by the inoculation of a guinea-pig with the spleen of the rat.

*Lesions in rats.*—The most constant and characteristic lesion of the plague-like disease in the white and the wild rat is a finely granular liver macroscopically indistinguishable from that so often observed in plague-infected rats. In addition there may be a fibrinous or necrotic area at the site of injection. The adjacent lymph nodes may be enlarged and congested but are not necrotic. The spleen is usually enlarged and dark, and occasionally granular. An intense subcutaneous injection may be observed. When the injection, the granular liver, and the large dark spleen coexist, the appearance is strikingly like plague.

*Susceptibility of cats and dogs.*—Two young dogs and four cats were inoculated subcutaneously with large doses of an emulsion made from the liver of a guinea-pig dead of this disease. The animals all remained well, while a control guinea-pig inoculated cutaneously with the same material died on the seventh day and

presented at autopsy the usual lesions. A white rat vaccinated with the emulsion remained well.

*Susceptibility of the desert ground squirrel.*—The desert ground squirrel (*Ammospermophilus leucurus*)<sup>1</sup> is a small rodent found widely distributed in the southern part of California. Two of these animals were vaccinated with spleen from a guinea-pig dead on the fifth day after inoculation from a naturally infected ground squirrel. One died on the fifth day, the other on the sixth. Both presented at autopsy bilateral axillary buboes, and granules in the liver and in the spleen. A ground squirrel (*Citellus beecheyi*) control died on the eighth day and presented the usual lesions of the plague-like disease.

*Susceptibility of farm animals.*—We have endeavored to determine the susceptibility of calves, sheep, swine, and goats. In the first experiment an emulsion was made by grinding in a mortar 1 gm. of spleen tissue from a guinea-pig dead of the disease (strain 1,059) and making a suspension in 10 c.c. of normal salt solution. One of each species was given 2 c.c. of the emulsion subcutaneously. A control guinea-pig inoculated by scarification with one loopful of the same material died on the sixth day with typical lesions. The calf, swine, and goat showed no effect from the inoculation. The sheep sickened, refused food, and died on the seventh day. At autopsy there was a bloody and somewhat gelatinous exudate at the site of injection and on the front of the thorax. The spleen was enlarged. A guinea-pig inoculated subcutaneously with a pea-sized fragment of the spleen died on the fourth day; a guinea-pig vaccinated from the spleen died on the seventh day; and one vaccinated from the gelatinous edema died on the sixth day. All had typical lesions.

To determine the persistence of the infectious agent in the bodies of the animals, material was aspirated from the site of inoculation on the days shown in the following table. A large hypodermic syringe was loaded with about 1 c.c. of physiological salt solution. This was injected into the tissue at the site, sucked back into the syringe, the needle withdrawn, and the material used to inoculate

<sup>1</sup> We are indebted to Mr. H. S. Swarth of the Department of Zoölogy, of the University of California, for the identification of this rodent.

a guinea-pig. The figures in the table show the day of death of the rodents. All died of the plague-like disease. The minus sign indicates that the test animals survived.

GUINEA-PIGS INOCULATED WITH MATERIAL ASPIRATED ON VARIOUS DAYS FROM FARM ANIMALS.

	1st Day 24 hours	2d Day	3d Day	4th Day	5th Day	7th Day	9th Day	12th Day	15th Day	19th Day
Calf.....	—	6	5	8	—	—	—	—	—	—
Swine.....	5	4	4	7	4	7	6	7	—	6
Sheep.....	5	—	7	4	5	Sheep dead	—	—	—	—
Goat.....	5	5	7	—	—	7	—	—	—	—

The results of the aspirations indicate that the calf carried the infectious agent until the fourth day, the pig until the 19th day, the goat until the seventh day, and the sheep until its death on the seventh day. Certain irregularities in the table probably indicate failure to reach the seat of infection with the needle used to withdraw the fluid.

In another series a salt solution emulsion was made of the liver, spleen, and bubo of a guinea-pig dead on the seventh day of the plague-like disease (strain 2,016). Four animals—a hog, a sheep, a goat, and a calf—received subcutaneously 2 c.c. of this suspension. A control guinea-pig which was inoculated cutaneously with the same material died on the seventh day with typical lesions. The hog and the sheep were negative. The goat developed an abscess at site. A guinea-pig inoculated subcutaneously with pus from the abscess developed a slough but recovered. The sheep died on the fourth day and presented the following lesions: Marked injection at site and a very small infiltration; a reddened, almost hemorrhagic area over front of sternum extending well down over the belly; below this an area of intense injection; a few black points on the surface of spleen; and considerable quantity of bloody, turbid fluid in pericardium. Guinea-pigs were inoculated by the cutaneous method with material from the site, from bubo, from spleen, and with heart's blood, respectively. The guinea-pig inoculated from the site died on the seventh day, presenting the usual lesions of this disease; the others survived.

To further test the susceptibility of sheep to this disease, three were inoculated. One was given a large dose and one a very small dose subcutaneously of emulsion of tissue of a guinea-pig (strain, 2,016), another was vaccinated with the spleen. The sheep that received the large dose sickened but recovered; the others remained well. The two guinea-pig controls that were inoculated subcutaneously with the emulsion died on the seventh and ninth days, respectively, with typical lesions.

#### NATURE OF CAUSATIVE AGENT.

*Thermal death point.*—The thermal death point had been found to be between 55° C. and 60° C. We have determined it within narrower limits than this. An emulsion was made of tissue (liver) from a guinea-pig dead on the sixth day after inoculation. Five-tenths of a c.c. of the suspension was placed in each of several drawn out glass pipettes. These were sealed at one end and immersed in a water bath for 10 minutes at the temperature shown in the following table. The heated material was then used to inoculate a guinea-pig. The maximum and minimum temperatures recorded by the thermometer during the 10 minutes that the emulsion was being heated are noted.

Range of Temperature ° C.	Fate of Guinea-Pig Inoculated with Heated Emulsion
53.8–54.2.....	Died sixth day; usual lesions
54.8–55.2.....	Died seventh day; usual lesions
55.8–56.2.....	Remained well
56.8–57.2.....	Remained well
57.8–58.0.....	Remained well

The results of this experiment indicate that heating to approximately 56° C.<sup>1</sup> for 10 minutes is sufficient to destroy the infective agent.

*To determine whether there is a spore stage.*—An emulsion of the tissue of a guinea-pig dead of the disease was mixed with enough sand to form a paste. This was permitted to stand in the laboratory exposed to the light for 48 hours. At the end of this period a suspension was made in saline solution of the sand-tissue mixture,

<sup>1</sup> We found that different thermometers of the same lot varied as much as 2° C. in their readings. The readings given here are those of the thermometer that read about midway between two others.



and 1 c.c. was injected into a guinea-pig. Another portion was heated in the water bath at 60°-70° C. for 10 minutes, and then injected into a guinea-pig. The animal that received the unheated suspension died of the plague-like disease on the fourth day. The one inoculated with the heated material remained well. We believe that this experiment justifies us in concluding that the organism as it exists in the tissues does not develop spores.

*Filtration experiments.*—Four Berkefeld filters were tested as to permeability by *B. coli*. Plates seeded with the filtrate from three of these remained sterile. Portions of an emulsion of organs of a guinea-pig dead of the plague-like disease were passed through each of these three filters and each filtrate was used to inoculate a guinea-pig subcutaneously. These animals remained well. The fourth filter permitted the fluid to pass through turbid and a guinea-pig inoculated with this material died on the fourth day with typical lesions.

*Degree of septicemia.*—In order to determine approximately the degree of septicemia in animals dead of the disease, we inoculated a series of guinea-pigs with varying doses of the heart's blood of a guinea-pig dead on the fifth day, and another series with the heart's blood of a squirrel dead on the eighth day after inoculation. In each case 0.0001 c.c. and 0.000001 c.c. reproduced the disease but 0.00000001 c.c. failed to convey the infection.

*Relation to plague immunity.*—On account of the close anatomical similarity to plague, it seemed worth while determining whether an immunity against plague protected against this disease. A guinea-pig which was inoculated subcutaneously on August 31, 1910, with bubo material from a case of human plague became very ill, but recovered. On November 27, 1910, this guinea-pig was vaccinated with a known highly virulent culture of the plague bacillus, but without any apparent effect. On February 3, 1911, it was inoculated subcutaneously with an enormous dose of a virulent culture of *B. pestis* but suffered no inconvenience. Twenty-two days later this animal was vaccinated with tissue from a ground squirrel dead of the plague-like disease. The guinea-pig died on the seventh day, presenting the usual lesions at autopsy.

## TRANSMISSION IN NATURE.

The earlier work left much doubt as to the mode in which this disease is conveyed from squirrel to squirrel under natural conditions, and numerous additional experiments have not served to solve this problem definitely.

*Feeding experiment.*—It had been shown that the disease may be conveyed by feeding, and the following experiment appears to indicate that infection is very readily brought about in this manner. Two guinea-pigs and three squirrels were fed carrots mixed with the organs of a squirrel dead of the plague-like disease. Both of the guinea-pigs died on the fifth day. In each case there was a bubo in the neck and the other usual lesions. One of the guinea-pigs showed in the wall of the intestine numerous yellowish-white nodules varying in size from a pin point to 1 mm. in diameter. One of the ground squirrels died on the seventh day. A bubo was found at the base of the mesentery; none elsewhere. The usual lesions were found in the liver and in the spleen. The second squirrel died on the eighth day, and the third on the 19th day. Buboes were found in the neck of each rodent and the other characteristic appearances were present. In one there were large caseous masses at the base of the mesentery, and the wall of the intestine showed numerous small whitish nodules.

*Lack of infectiveness of feces.*—On two occasions we have taken fresh feces from cages in which guinea-pigs had died of the disease, made an emulsion of this material, and used it to inoculate guinea-pigs. In each case the result was negative.

*Infection due to gross contamination of cages.*—We have also failed to convey the infection through washings of the cage litter (feces, sawdust, etc.) from containers in which animals had been kept and had died of the disease. These results are in harmony with the fact that unless fleas were present we have never seen infection occur when healthy animals—ground squirrels or guinea-pigs—were placed in the cages with sick ones and kept there long after the death of the latter.

It seemed worth while determining whether heavy contamination of the cage with organs from a rodent dead of the disease

would lead to the infection of healthy animals. The spleen and liver of a guinea-pig, dead on the fourth day, were ground in a mortar, thoroughly mixed with sawdust, and placed in a cage with two ground squirrels. These animals both died, one on the eighth and the other on the 20th day. The lesions were typical in both rodents. Judged by the location of the bubo which in each case was in the neck, we consider it probable that the infection was transmitted by the ingestion of contaminated material. The presence of peripheral buboes in locations other than the neck leads us to believe that the disease is not contracted in nature<sup>1</sup> through feeding.

*Flea transmission.*—In the earlier paper, the infection of guinea-pigs with crushed fleas taken from rodents dead of the disease occurred only when the inoculations were made within 24 hours after the removal of the parasite from the host. We have made more extensive experiments both with squirrel fleas (*C. acutus*) and rat fleas (*C. fasciatus*) and have found that the insects may harbor the infection for a longer period. In each case fleas were placed in the cage with guinea-pigs inoculated with tissue from an animal dead of the disease and allowed to remain there until the animal died. As many fleas as possible were secured from the dead rodent and from the cage. Four of these were crushed and used to inoculate guinea-pigs at once after their removal from the dead host, and four (live) on three succeeding days. All of the rodents died that were inoculated with rat fleas immediately after they were taken from the dead guinea-pig. Two of those inoculated after 24 hours died, while only one of those inoculated at the end of 48 hours succumbed. The others remained well.

The experiment with squirrel fleas was carried out in the same manner. Two of the guinea-pigs inoculated at once with insects taken from the dead host perished, while none died that were inoculated on later days. Some of the guinea-pigs in this series developed what we regard as a chronic form of the disease.

In the preceding paper two cases were reported that might be regarded as probable examples of flea transmission. In each case ground squirrels infected with the disease were placed in cages

<sup>1</sup> See *Jour. Hyg.*, 1906, 6, p. 425, for the discussion of this subject in relation to plague.

with an abundance of squirrel fleas (*C. acutus*). Healthy squirrels placed in the cage with the sick developed the disease. It is obvious that such evidence is not conclusive but it is entitled to some weight.

We present here an experiment that proves conclusively that fleas may transmit the infection.

On January 21, 1911, a ground squirrel was vaccinated from the liver of a guinea-pig dead on the fifth day. The next day a large number of fleas (*C. acutus*) were placed in the cage with this squirrel. The rodent died on the seventh day and about 100 fleas were collected from the body. These fleas were placed in a clean cage with a healthy squirrel. The latter died 15 days later and presented the usual lesions of the plague-like disease, the bubo being in the neck. While there can be no doubt that this squirrel was infected by the fleas we are not sure that this is the usual means of transmission of the disease in nature.

We have made many attempts to carry the infection from one guinea-pig to another by means of squirrel fleas and rat fleas, but without success. We have also made a number of unsuccessful attempts to carry it by squirrel fleas from one squirrel to another. The failure of attempts to demonstrate the infection in feces and in cage washings, the failure of mere contact with infected rodents, the success of contact in the presence of fleas, and the one unimpeachable flea transmission experiment lead us to believe that these insects are concerned in the propagation of the disease.

#### A NOTE ON THE CAUSATIVE AGENT, BACTERIUM TULARENSE.<sup>1</sup>

When smears from the spleen of a guinea-pig or a ground squirrel dead of this disease are stained with carbol fuchsin or aniline gentian violet, there will often be found large numbers of round or rod-shaped bodies usually lying inside well-defined, round, or oval clear areas. Several of these may occur within one clear space which for the sake of convenience will be termed the capsule. What appear to be these same rods minus the capsule are often found in the leukocytes. These objects, which we regard as the

<sup>1</sup> From Tulare, the county in California in which the disease was first observed.

essential cause of this disease, are very minute. The size is approximately as follows: Length of rod,  $0.3\ \mu$  to  $0.7\ \mu$ , length of capsule  $0.4\ \mu$  to  $1\ \mu$ ; breadth of organism  $0.2\ \mu$ ; breadth of capsule  $0.3\ \mu$  to  $0.5\ \mu$ . With methylene blue this organism stains very poorly and shows no capsule. These encapsulated rods are found in enormous numbers in the spleen of nearly all guinea-pigs and squirrels, dead of this disease. The liver, bubo, and heart's blood usually contain the organisms, but in much smaller numbers than the spleen.

Attempts to cultivate the organism are not uniformly successful, and it is evident that we have not yet found the conditions best suited for its development. We have at present the seventh generation on Dorset's egg medium, the only culture material on which we have obtained a growth. A visible culture appears in three to five days in the form of transparent coalescent drop-like colonies. Guinea-pigs vaccinated with the first, second, and third generations died, presenting typical gross and microscopical appearances of the plague-like disease. Guinea-pigs vaccinated with the fourth and fifth generations developed a subacute form of the disease.

The morphology of the organism grown on artificial media is much like that described above. There is considerable apparent involution, enlargement, and irregularity of form. In some cultures large globular forms predominate. Unless the culture is mixed with something (serum) capable of giving a staining background the evidence of a capsule is usually not apparent. The microscopical appearance of smears from cultures as well as those from organs is very characteristic. In both cases the organism stains very poorly with methylene blue. It is apparently non-motile.